Primary Cutaneous Marginal Zone B-Cell Lymphomas Are Targeted by Aberrant Somatic Hypermutation

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A mechanism inducing genetic instability, termed aberrant somatic hypermutation (ASHM), has been described in diffuse large B-cell lymphoma. To further investigate whether ASHM also occurs in primary cutaneous marginal zone B-cell lymphoma (PCMZL), we studied the mutational profile of *PAX5, RhoH/TTF, cMYC, and PIM1* in 11 PCMZLs. A total of 17 sequence variants were found in 8 of 11 lymphomas cases (72.7%), and they displayed the molecular features typical for the ASHM. Further, two mutations, one mutation in *PIM1* and one in *cMYC*, led to amino-acid substitution with potential functional consequences. These data indicate that ASHM is associated with PCMZLs. By mutating regulatory and coding sequences of the targeted genes, ASHM may represent a major contributor to their pathogenesis.

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INTRODUCTION

The pathogenesis of most human B-cell non-Hodgkin's lymphomas is associated with distinct genetic lesions, including chromosomal translocations and aberrant somatic hypermutation (ASHM), which arise from mistakes during class switch recombination and somatic hypermutation occurring in the germinal center (Küppers and Dalla-Favera, 2001; Pasqualucci *et al.*, 2001). Somatic hypermutation occurs in germinal center B cells and is found in all germinal center-derived B-cell tumors. This process was thought to selectively target the rearranged *V* genes to generate antibody diversity. Activation-induced cytidine deaminase (AID) is an enzyme required for both reactions, and mistargeting of AID to known proto-oncogenes linked to B-cell tumorigenesis in germinal center B cells combined with a breakdown of protective high-fidelity repair mechanism has been shown to be a principal contributor to the pathogenesis of B-cell non-Hodgkin's lymphoma (Liu *et al.*, 2008; Pasqualucci *et al.*, 2008). In diffuse large B-cell lymphoma somatic hypermutation aberrantly targets the 5′ sequences of several proto-oncogenes relevant to lymphomagenesis, including *PIM1*, *PAX5*, *RhoH/TTF*, and *cMYC*. This phenomenon, termed ASHM, occurs in >50% of diffuse large B-cell lymphoma, but is rare in indolent lymphomas (Pasqualucci *et al.*, 2001; Gaidano *et al.*, 2003; Bodor *et al.*, 2005; Dijkman *et al.*, 2006; Deutsch *et al.*, 2007; Halldorsdottir *et al.*, 2008).

Primary cutaneous marginal zone B-cell lymphoma (PCMZL) is one of the most frequent types of cutaneous B-cell lymphomas. PCMZL, an indolent lymphoma, is considered as part of the group of extranodal marginal zone B-cell lymphoma involving mucosal sites termed MALT (mucosa-associated lymphoid tissue) lymphomas (Willemze *et al.*, 2005). PCMZL shares some histological and clinical features with MALT lymphoma of extracutaneous origin, but differences in biology with respect to dissemination, association to chronic inflammation, and treatment modalities are found between PCMZL and extracutaneous MALT lymphomas, suggesting that they should be considered as separate entity (Rijlaarsdam *et al.*, 1993; Cerroni *et al.*, 1997a, b; Fink-Puches *et al.*, 2002). The present study was aimed at investigating the role of the ASHM in PCMZL.

RESULTS

A total of 11 samples of PCMZLs were subjected to DNA sequence analysis of *PAX5, RhoH/TTF, cMYC, and PIM1* (Table 1). Of 11, 8 (72.7%) lymphoma samples showed somatic mutation in at least one of the four analyzed genes. Each of the four genes investigated was altered in a significant fraction (Table 2): *PAX5* was mutated in 2 of 11 (18.2%) samples, *RhoH/TTF* in 3 of 11 (27.3%), *cMYC* in 4 of 11 (36.4%), and *PIM1* in 5 of 11 (45.5%) samples. To confirm somatic nature of these mutations, corresponding germ-line DNA was sequenced in selected cases (corresponding to cases 3, 10, and 11), demonstrating the tumor-specific origin.

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Abbreviations: AID, activation-induced cytidine deaminase; ASHM, aberrant somatic hypermutation; bp, base pair; PCMZL, primary cutaneous marginal zone B-cell lymphoma

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To rule out the occurrence of mutations in benign B cells, *Borrelia burgdorferi*-infected skin lesions that are rich in inflammatory B cells were included showing no mutations.

The detailed characterization of the mutational profile is summarized in Table 1 and their features in Table 2. In total, 17 sequence variants were found. These mutations were exclusively single base pair (bp) substitutions. The average frequency of mutations, calculated taking into account only mutated cases, ranged from 0.02×10^{-2} per bp in the case of *cMYC* to 0.13×10^{-2} per bp in the case of *RhoH/TTF*; taking all four genes together the average frequency of mutation was 0.07×10^{-2} per bp (Table 2). Of the 17 mutations, 15 were transitions and 2 were transversions corresponding to a transition-to-transversion ratio of 7.5 (expected 0.5, if each nucleotide is targeted at identical rate). In addition, the ratio of G+C to A+T was shifted to a clear predominance of G+C mutations. Four of five mutations in *PIM1* and four of five mutations in *cMYC* were located in the coding region, one mutation (threonine for proline in *PIM1* gene and asparagine for aspartic acid in *cMyc* gene) in each gene led to amino-acid substitution with potential functional consequences (Table 1).

Table 1. Mutational analysis of *PAX5*, *RhoH/TTF*, *cMYC*, and *PIM1*

	PAX5	RhoH/TTF	cMYC	PIM1
Case 1				A 1414 G
Case 2				
Case 3			C 4655 T	**A 1237 C**
Case 4		T 415 C A 449 G		
Case 5				
Case 6	G 882 A			C 1303 T
Case 7			T 3493 C	C 1306 T
Case 8				
Case 9		T 936 C		
Case 10			**A 4608 G** C 4686 T	G 1938 A
Case 11	C 1104 T	C 945 T G 981 A	C 4693 T	

Bold characters represent missense mutations.

DISCUSSION

In PCMZL, molecular analyses show a number of distinct cytogenetic alterations in <50% of cases (Cerroni, 2006), but a molecular mechanism inducing genetic instability has not yet been described. We show that 72% of PCMZL lymphoma samples show point mutations in at least one of the four analyzed proto-oncogenes. Although only a few PCMZL cases were mutated at the loci of *PAX5*, nearly 46% of cutaneous MALT lymphoma cases harbored mutations in the *PIM1* gene and >36% in the *cMYC* gene locus.

Several features of the molecular profile of mutations—like predominance of GC over AT mutations; elevated transition over transversion ratio, frequency of mutations and their distribution 1–2 kb downstream of the transcription initiation site—are similar to that detected in other lymphoma entities, and closely resemble the one reported for *IgV* and *BCL6* mutations (Pasqualucci *et al.*, 1998, 2001; Shen *et al.*, 1998; Storb *et al.*, 1998; Peng *et al.*, 1999; Gaidano *et al.*, 2003; Rossi *et al.*, 2005; Deutsch *et al.*, 2007), suggesting ASHM to be responsible for these nucleotide alterations. Further, mutations at these four loci have not been found in normal germinal center B cells in diffuse large B-cell lymphoma (Pasqualucci *et al.*, 2001) or in inflammatory B cells from *B. burgdorferi*-infected skin lesions, supporting the hypothesis that these mutations are tumor specific and result from an abnormal activity of the SHM mechanism. Besides primary cutaneous follicle center lymphomas (Dijkman *et al.*, 2006), PCMZLs are the second cutaneous lymphoma entity with indolent clinical course to be aberrantly targeted in these four proto-oncogenes. However, although the mutational load analyzed for all genes is higher in primary cutaneous follicle center lymphoma (mutation frequency 0.1 vs 0.07 per 100 bp) compared to PCMZL, more PCMZL cases (72 vs 53%) are affected by ASHM.

Because the genes targeted by ASHM are proto-oncogenes, introducing somatic mutations may have important functional consequences. First, mutations clustering around the 5′-regulatory regions may deregulate gene transcription as previously documented for *cMYC* (Cesarman *et al.*, 1987). Second, in the case of *cMYC* and *PIM1* in which coding sequences are also targeted causing amino-acid substitutions, the mutations may alter structure and, subsequently, the function of these molecules. Mutations affecting the *cMYC* transactivation domain can alter the functional properties of *cMYC* by interfering with its phosphorylation, protein

Table 2. Distribution and feature of *PAX5*, *RhoH/TTF*, *cMYC*, and *PIM1* mutations in PCMZL

Locus	Mutated cases/ tested (%)	Mutation frequency per 100 bp (range)[1]	Single base pair substitution	G+C//A+T	Transitions over Transversions
PAX5	2/11 (18.2)	0.097 (0.096–0.098)	2	2//0	2//0
RhoH/TTF	3/11 (27.3)	0.12 (0.10–0.13)	5	2//3	5//0
cMYC	4/11 (36.4)	0.025 (0.02–0.04)	5	3//2	4//1
PIM1	5/11 (45.5)	0.05 (0.04–0.06)	5	3//2	4//1
All genes	8/11 (72.7)	0.07	17	10//7	15//2

[1]Mutation frequencies were calculated on the entire region analyzed and on mutated cases only, considering two alleles per gene per case.

stability, or repression of transactivation by the RB-related protein p107 (Cesarman *et al.*, 1987; Raffeld *et al.*, 1995).

In diffuse large B-cell lymphoma, 17 of 130 (13%) investigated genes were found involved in ASHM (Pasqualucci *et al.*, 2004), suggesting that a much higher number of genes might be affected by this mechanism and that AID-mediated mutations outside the *IgV* regions are a genome-wide phenomenon (Wang *et al.*, 2004; Liu *et al.*, 2008). Although the mutational profile of *PAX5, RhoH/TTF, cMYC, and PIM1* of PCMZL is similar to the mutational profile of extracutaneous MALT lymphomas (Deutsch *et al.*, 2007), a different targeting of additional genes by ASHM might contribute to the different biological behavior between PCMZL and extracutaneous MALT lymphomas (Rijlaarsdam *et al.*, 1993; Cerroni *et al.*, 1997a, b; Fink-Puches *et al.*, 2002).

Recently, it has been demonstrated that *Helicobacter pylori* infection—a risk factor for the development of gastric cancer—triggers aberrant expression of AID in gastric epithelium and *H. pylori*-mediated upregulation of AID resulted in the accumulation of nucleotide alterations in the TP53 tumor-suppressor gene (Matsumoto *et al.*, 2007). As PCR analyses show the presence of *B. burgdorferi* infection in roughly 20% of cases of PCMZL (Cerroni, 2006), it is tempting to speculate whether a similar mechanism is responsible for some of the mutations observed in our study.

In conclusion, we demonstrate that in the majority of PCMZLs, the proto-oncogenes *PAX5*, *RhoH/TTF*, *c-MYC*, and *PIM1* are targeted by ASHM. These molecular changes may be of functional relevance in the development of PCMZLs. By mutating regulatory and coding sequences of the targeted genes ASHM may represent a major contributor to their pathogenesis.

MATERIALS AND METHODS

Materials, diagnoses, and DNA extraction

DNA extraction (DNA Mini Kit; Qiagen, Valencia, CA) was performed from formalin-fixed and paraffin-embedded macrodissected tissue containing at least 90% malignant cells. A total of 11 PCMZL specimens, 3 *B. burgdorferi*-infected skin lesions, and 4 normal controls, including 3 nonneoplastic tissues samples of selected lymphoma cases (corresponding to cases 3, 10, and 11), and 1 peripheral blood mononuclear cells were included. Ethical approval was obtained from local hospital ethics committee in accordance with Declaration of Helsinki Principles. All lymphomas were classified according to the European Organization for Research and Treatment of Cancer, World Health Organization classification for cutaneous lymphomas (Willemze *et al.*, 2005). All specimens were obtained from the tissue bank of the Institute of Dermatology.

Sequencing analysis of PAX5, RhoH/TTF, c-MYC, and PIM1

Mutational profile by direct DNA sequencing of *PAX-5* (AF386790S2 (exon 1B):630–1450) *RhoH/TTF* (AF386789 (exon 1): 265–1009), *c-MYC* (X00364 (exon 1 through 2): 2289–3626 and 4486–5068), and *PIM1* (AF386792 (exon 1 through 4): 780–2080) was generated on selected regions previously described to contain >90% of mutations (''mutational hot spots'') found in diffuse large B-cell lymphoma (Pasqualucci *et al.*, 2001). The initial PCR was performed using the HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany), and all oligonucleotides were also used for sequencing (available in Table S1). PCR products were purified and sequenced from both sides using the BigDye terminator chemistry 3.1 (Applied Biosystems, Foster City, CA). Sequences were run on an ABI3730 automated sequencer (Applied Biosystems). Sequence variants were confirmed by two independent PCR reactions. Nucleotide changes corresponding to previously published polymorphism were excluded from analysis. Further, all changes occurring more than once in separate cases were considered as polymorphic variants and were disregarded.

Statistical analysis

Statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, IL). To calculate differences between the data set of PCMZLs and the previously published mutational profile of extracutaneous MALT lymphomas (Deutsch *et al.*, 2007) Student's *t-test* was used.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Primers used for the amplification of PAX5, RhoH/TTF, cMYC, and PIM1

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